NAVAL MEDICAL RESEARCH INSTITUTE

Bethesda, MD 20889-5055

NMRI 90-61

August 1990

ALCALIGENES EUTROPHUS AS A SOURCE OF HYDROGENASE: AN EVALUATION OF TECHNIQUES FOR ITS LARGE SCALE PRODUCTION

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NMRI 90-61

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SECURITY CLASSIFICATION OF THIS PAGE

AD-A226 476

REPORT DOCUMENTATION PAGE

1a. REPORT SECURITY CLASSIFICATION UNCL	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT Approved for public release; distribution is unlimited
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S) NMRI 90-61	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION Naval Medical Research Institute 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION Naval Medical Command
6c. ADDRESS (City, State, and ZIP Code) 8901 Wisconsin Avenue Bethesda, MD 20814-5055	7b. ADDRESS (City, State, and ZIP Code) Department of the Navy Washington, DC 20372-5120
8a. NAME OF FUNDING/SPONSORING ORGANIZATION Naval Medical Research & Development Comman 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER
8c. ADDRESS (City, State, and ZIP Code) 8901 Wisconsin Avenue Bethesda, MD 20814-5044	10. SOURCE OF FUNDING NUMBERS PROGRAM ELEMENT NO. MR00001 / PROJECT NO. .001 / TASK NO. / WORK UNIT ACCESSION NO. 1376

11. TITLE (Include Security Classification)
(U) ALCALIGENES EUTROPHUS AS A SOURCE OF HYDROGENASE: AN EVALUATION OF TECHNIQUES FOR ITS LARGE SCALE PRODUCTION

12. PERSONAL AUTHOR(S)
K. K. Kumaroo and G. J. Imbert

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Technical Report	FROM 1989 TO 1990	1990 July	53

16. SUPPLEMENTARY NOTATION

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	hydrogen diving, biochemical decompression, hydrogenase

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

Alcaligenes eutrophus H16 (ATCC 17699), an aerobic H_2 oxidizing bacteria, has been selected as the most suitable enzyme source for a NAD-linked soluble hydrogenase. The hydrogenase will be used to study the feasibility of biochemical decompression in hydrogen dive experiments. The choice of this enzyme was based on its ability to oxidize molecular hydrogen to water and its exceptional O_2-resistance under general physiological conditions.

Methods for growing A. eutrophus in the absence of externally supplied hydrogen have been reviewed with special consideration for their adaptability for mass production of the microorganism under safe conditions. Two methods that avoid the handling of large quantities of inflammable H_2 mixtures have been compared and tested in this pilot study. One was the chemolithotrophic method of Schuster & Schlegel, which involved the cultivation of the bacteria in a mineral medium under electrolytically produced

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT ☐ OTIC USERS	Unclassified
22a. NAME OF RESPONSIBLE INDIVIDUAL Regina E. Hunt, Command Editor	22b. TELEPHONE (Include Area Code) (202) 295-0198 / 22c. OFFICE SYMBOL SD/RSD/NMRI

DD FORM 1473, 84 MAR — 83 APR edition may be used until exhausted. All other editions are obsolete.

SECURITY CLASSIFICATION OF THIS PAGE
UNCLASSIFIED

H_2 and O_2, and externally supplied CO_2; the other was the heterotrophic procedure of Friederick and coworkers, which avoided H_2 altogether but supplied the culture medium with fructose and glycerol as energy and carbon sources. Because of its simplicity, rapidity, high yield, and easy adaptability for large scale production, the heterotrophic method was selected. This method is expected to provide the required amount of enzyme for further development of the current research project.

ACKNOWLEDGEMENTS

This work was supported by Naval Medical Research and Development Command Research No. MR00001.001-1376. The opinions or assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

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1. OBJECTIVES.

This report deals with the identification of appropriate hydrogen bacteria, as well as the development of suitable technique to cultivate them on large scale in order to extract and purify the required quantity of the hydrogenase enzyme. This enzyme will be employed as a biochemical decompression agent during the first phase of animal experiments using hydrogen diving.

1.1. General outline of the project.

Prevention of decompression sickness relies upon the slow release and elimination of the dissolved gases in the diver's tissues by a physical process during ascent. The basic flaw of this method is that the diver's tissues are supersaturated with gas during ascent, in order to provide the driving force for its elimination into the ambient milieu (used as the gas sink) down a pressure concentration gradient. As supersaturation is the basic cause of decompression sickness, current ascent procedures cannot be considered as completely safe.

Biochemical decompression could be an alternate method for insuring faster and safer diver ascent rates. This method would rely on the chemical elimination of gases within the body, using the tissues as the gas sink. In order to accomplish this, the diving gas must be amenable to biochemical manipulation. The two major components of the currently used diving mixtures are helium and nitrogen. Both are beyond biochemical manipulation since helium is chemically inert on one hand, while on the other hand the potential reaction products of nitrogen would be highly toxic to the diver.

It has now been demonstrated that hydrogen (H_2) may be substituted for helium or nitrogen, and was found to be especially valuable in deep dives (31,32). H_2 may be enzymatically oxidized by oxygen (O_2) in the tissues and eliminated as water from the body. This would only require the presence in the tissues of the enzyme "hydrogenase" which can catalyze the oxidation of hydrogen to water. However very little is known about the presence of such an enzyme in mammalian system, but it is widely assumed that human tissues lack the ability to metabolize molecular hydrogen. Therefore, it is proposed to present the tissue with the enzyme hydrogenase (conveniently obtained from hydrogen oxidizing

bacteria that are widely distributed in nature), thus enabling the tissue to oxidize the dissolved hydrogen and eliminate it as water.

1.2. Specific objectives of the present study.

A demonstration of the feasibility of biochemical decompression is therefore contingent upon the identification of a suitable bacteria and obtaining the critical enzyme from it in a form that can be safely and efficiently administered to experimental subjects.

The specific objectives of the present study were:

a) to identify an appropriate microorganism as a source of a hydrogenase enzyme that can function under the physiological conditions that prevail in mammalian tissues (O_2 pressure, ionic composition, pH, temperature etc.)

b) to select a suitable method to grow this microorganism on large scale, in order to extract and purify substantial amounts of active hydrogenase to initiate the preliminary experiments in laboratory animals (Wistar rats).

2. SELECTION OF *Alcaligenes eutrophus* AS THE HYDROGENASE SOURCE.

2.1. Hydrogen-oxidizing bacteria: Historical aspects.

H_2 oxidization by some unknown agent present in the soil was detected as early as the middle of 19th century, but that this process was mediated by a microorganism became evident only at the beginning of this century. Kaserer (in 13) reported isolation of an autotrophic bacteria (*Hydrogenomonas pantotropha*) capable of living on energy liberated through oxidization of H_2 gas by O_2. The term "*Knallgasbacteria*" (52) was also used to describe this group of bacteria because of their ability to grow under H_2-O_2-CO_2 atmosphere. These hydrogen-oxidizing bacteria were once thought to form a taxonomic group and were classified under a special genus, "*Hydrogenomonas*", but this classification was later rejected because of increasing heterogeneity found among strains, and therefore

the individual species have been placed later under pre-existing genera of heterotrophic bacteria - such as *Alcaligenes, Pseudomonas* and so on.

Ever since Kaserer's discovery of the Hydrogen-oxidizing bacteria biochemists were in search of an enzyme system in these organisms, which catalyzed oxidation of molecular H_2. Finally in 1931, Stephenson and Stickland (14,15) demonstrated the existence in *Escherichia coli* of a "hydrogenase" enzyme which was able to catalyze the reduction of methylene blue by molecular H_2. Attempts to isolate hydrogenases and study their biochemical properties were largely unsuccessful for over 40 years thereafter. Due to the efforts by Schlegel and associates in Europe, and Mortenson and others in the United States the study of the physiology and biochemistry of hydrogen bacteria and of the regulation of the hydrogenase enzyme system eventually received an unprecedented boost in the scientific community. The three international symposiums (36,37,53) and numerous reviews on hydrogen metabolism and hydrogenases (13,17,18,19,21,42,51) indicate the growing interest in the biochemistry, genetics and biotechnological potentialities of hydrogen-oxidizing bacteria, particularly the hydrogenase enzyme systems in these microorganisms. It was significant to note that the 1980s witnessed new and exciting key discoveries in bacterial hydrogenase research, not the least of which include (i) identification of nickel as an essential component of a large number of hydrogenases; (ii) detailed characterization of the physical, spectroscopic and redox properties of metal clusters in these enzymes; (ii) preliminary crystallographic studies; (iv) the cloning and sequencing of hydrogenase genes from several diverse species of bacteria; and (v) biotechnological exploitation of the enzyme for industrial use.

2.2. Hydrogenases: Occurrence and general characteristics.

The term "hydrogenase" refers to enzymes that catalyze the consumption or evolution of molecular hydrogen according to the reaction:

$$H^2 \rightleftharpoons 2H^+ + 2e^-$$

All hydrogenases are bidirectional to some extent in vitro, but the enzyme appears to catalyze only H_2 oxidation or reduction under physiological conditions. Hydrogenases have been found in a wide variety of micro-organisms (13,17,18,19,21,22), including anaerobic, aerobic and photosynthetic bacteria, and also in almost 50% of eukaryotic algae and in some facultative anaerobic protozoans (64). Their presence have also been reported in higher plants and in animal species (23). The basic processes of hydrogen utilization by various bacteria in the presence of different hydrogen acceptors are depicted in Figure 1.

Hydrogen evolution usually occurs in anaerobic microorganisms, and serves to get rid of excess reductant when protons are the only available oxidant (33). On the other hand, H_2 utilization or consumption can occur in aerobic as well as anaerobic bacteria, and is linked to ATP-producing electron transport systems. Anaerobic bacteria can oxidize H_2 using sulfate, sulfur, CO_2, nitrate etc. as the terminal electron acceptor, and the photosynthetic bacteria use H_2 and other compounds as the reductant for CO_2 fixation (35). Aerobic N_2-fixing bacteria evolve and consume H_2, and among this group are *Rhizobia*, the *Azotobacter* and the *Cynobacteria*. H_2 evolution by these micro-organisms are ATP-dependent and is catalyzed by nitrogenase but a hydrogenase is responsible for H_2 oxidation in these microorganisms.

A group of aerobic H_2-oxidizing bacteria and the hydrogenase enzyme system in them have received special attention during the last twenty years. This group of bacteria is strictly defined by their unique, ATP-independent enzymatic capability to utilize gaseous hydrogen as electron donor with oxygen as the final electron acceptor, and to fix carbon dioxide. These bacteria are different from those other aerobic organisms that can oxidize hydrogen but are unable to fix carbon dioxide autotrophically. These organisms are also different from the bacteria that utilize hydrogen under anaerobic conditions with sulfate, CO_2, etc. as hydrogen acceptors.

Hydrogenases isolated from diverse bacteria possess the fundamental property of reversible activation of hydrogen. Traditionally, hydrogenases have been broadly classified based upon their physiological roles either as 'uptake' - hydrogenases (responsible for utilization of molecular hydrogen

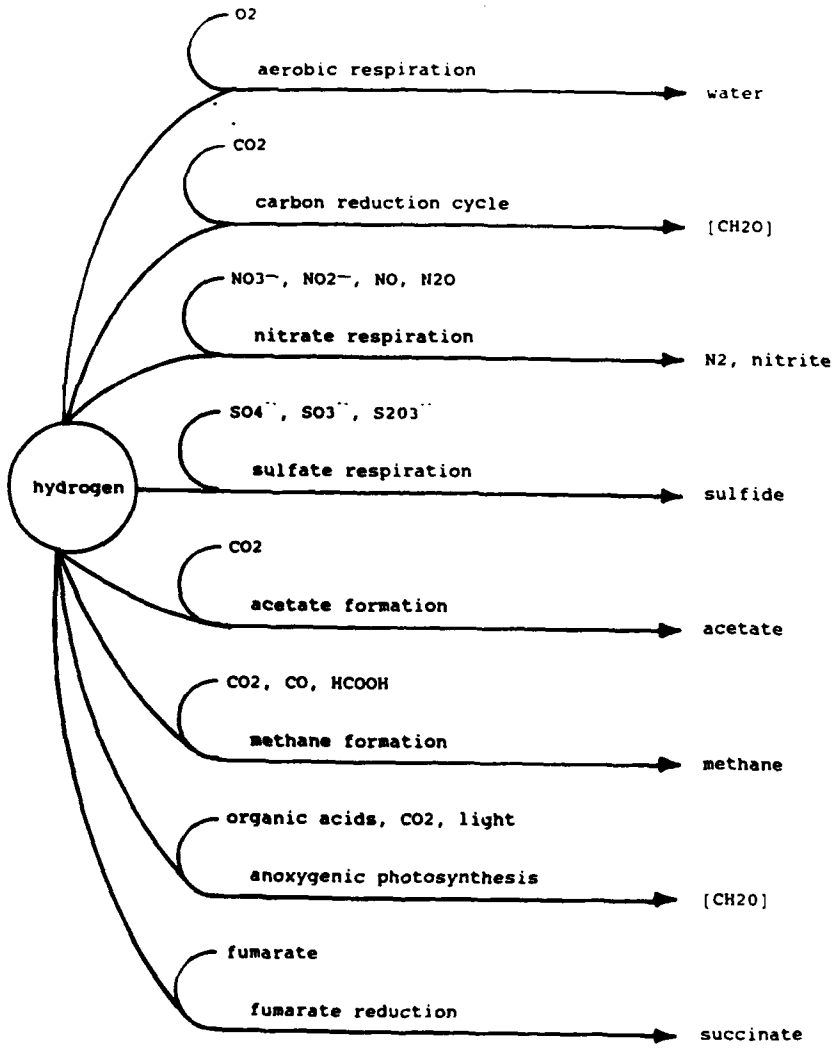


Figure 1. The basic processes of hydrogen utilization by various groups of bacteria in the presence of different hydrogen acceptors (54).

from the environment as an energy source) or as 'bidirectional' H_2 evolution hydrogenases employing the protons as the terminal electron acceptor. However, among genera, hydrogenases are very diverse with regard to their intra-cellular localization, structural and catalytic properties and metallic composition. Over a dozen hydrogenases from a diverse group of bacteria have been isolated, purified and characterized. They were all found localized either in the cytoplasm or in the membrane or both of the bacteria (18,22 and Appendix 1). The majority of the H_2-oxidizing bacteria contain a single membrane-bound hydrogenase while a few species, for example *Nocardia opaca*, contain only the cytoplasmic or 'soluble' enzyme. The aerobic H_2-oxidizing bacteria, *Alcaligenes eutrophus*, on the other hand contains both soluble and membrane-bound enzymes (8,17), and each enzyme serve two separate functions. The soluble enzyme catalyzes H_2-dependent NAD^+ reduction for CO_2-fixation and the membrane-bound enzyme is linked to electron-transport chain and energy production. (See Figure 2 for a diagrammatic representation of the soluble and membrane-bound hydrogenase of *A. eutrophus*.)

Hydrogenases isolated from different organisms differ greatly from each other but a common feature is that they all are metalloenzymes and contain non-haem iron and acid-labile sulfur in equivalent amounts. The iron-sulfur core (Figure 3) at the active center of the enzyme molecule consists of one or several [4Fe-4S], [3Fe-3S] and or [2Fe-2S] clusters, and these metal clusters are thought to be the redox-active component in these enzymes (24). In addition to iron-sulfur centers, spectroscopic and analytical studies revealed the presence of the transition metal nickel (4) and occasionally selenium (38) in the active center of several hydrogenase enzymes. On the basis of these studies and differences in catalytic functions, hydrogenases have been classified into several categories such as, protein containing nickel ([Ni-Fe]-hydrogenase), protein lacking nickel ([Fe]-hydrogenase), and protein containing selenium and nickel ([Ni-Fe-Se]-hydrogenase) etc. (39). Both nickel and selenium are believed to play a critical role in the synthesis of active hydrogenase (4,38). Hydrogenases that contain only iron-sulfur clusters are generally, but not always, susceptible to oxygen toxicity while [Ni-Fe]-hydrogenases are more oxygen resistant, as is the case with the soluble hydrogenase from *A. eutrophus*. By and large [Ni-Fe]-hydrogenases catalyze the consumption of hydrogen ('H_2-uptake' hydrogenase or Hup) while the

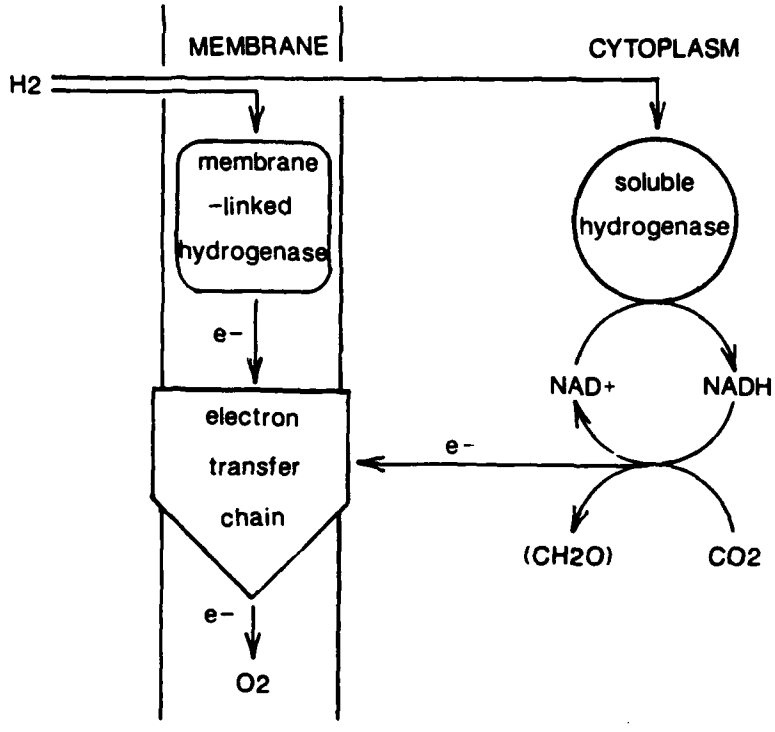
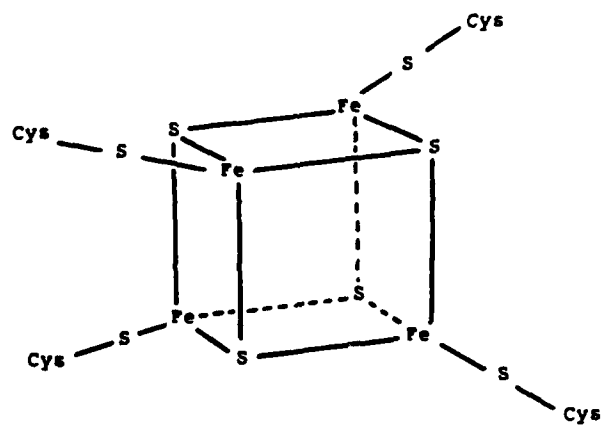


Figure 2. Schematic representation of the hydrogen oxidizing enzyme systems in *Alcaligenes eutrophus* (After Schlegel & Schneider 36): The membrane linked hydrogenase is considered to be the first component of the respiratory chain, where the electrons donated by molecular H_2 are first transferred to a primary acceptor, (possibly a quinone) and from there to the final acceptor O_2 - mediated by cytochromes. The primary function of the soluble hydrogenase is suggested to be the generation of NADH, used as the reducing power for CO_2 fixation, but it may as well channel electrons into the respiratory chain.

(i)



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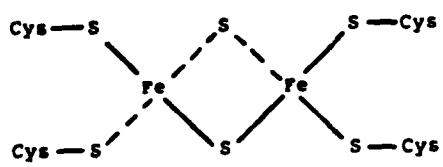

Figure 3. Schematic arrangement of iron and sulfur atoms in (i) [4Fe-4S] and (ii) [2Fe-2S] clusters.

[Fe]-only hydrogenases are involved in the production of hydrogen (Hpr). Most reports indicate that the maximum rate of H_2 production or consumption occur at acid and alkaline pH, respectively, as might be expected of reactions in which free protons are a substrate or product.

Based on kinetic, spectroscopic and isotope exchange data several reaction mechanisms have been proposed for the activation of hydrogen by the enzyme. Most of the models are of multicluster enzymes from strict anaerobes. However, there is a general consensus (17,24,25,55) that the catalysis involves binding of molecular hydrogen to a metal-sulfur cluster, followed by heterolytic cleavage of H_2 to produce a free proton and an enzyme-metal hydride (25,55). Cammack et al. (56,57) proposed a general working model (Figure 4) for the [Ni-Fe]-hydrogenase molecule to explain the role of nickel in the activation of hydrogen and to rationalize the available evidences on the reversible inactivation of the enzyme by oxygen.

The hydrogenase enzyme proteins are quite varied with respect to their molecular mass and sub-unit composition; however, the largest group now appears to be those with two (α and β) subunit composition, with a molecular mass approximately 60 and 30 KD each. Examples include the dimeric hydrogenases isolated from hydrogen bacteria [*Alcaligenes eutrophus* H16 (16), *Alcaligenes latus* (41)], sulfur bacteria [*Desulfovibrio gigas* (42)] and photosynthetic bacteria [*Rhodobacter capsulatus* (43)]. The membrane-bound hydrogenases from the aerobic N_2-fixing bacteria *Bradyrhizobium japonicum* (44) and *Azobacter vinelandii* (45) have also been shown to be dimers. The NAD-linked soluble hydrogenases of both *Alcaligenes eutrophus* and *Nocardia opaca Ib* have four non-identical subunits each (13,46), and have complex immunological and catalytic properties. Several other hydrogenases have only one subunit of varying molecular masses and their properties are still under investigation.

Immunological relationship among hydrogenases have been studied (47,48) and found a substantial degree of homology among the hydrogenases of a number of hydrogen-oxidizing bacteria.

2.3. Hydrogenases: Oxygen sensitivity.

As an electron acceptor of a high positive redox potential, oxygen enables cells to channel the

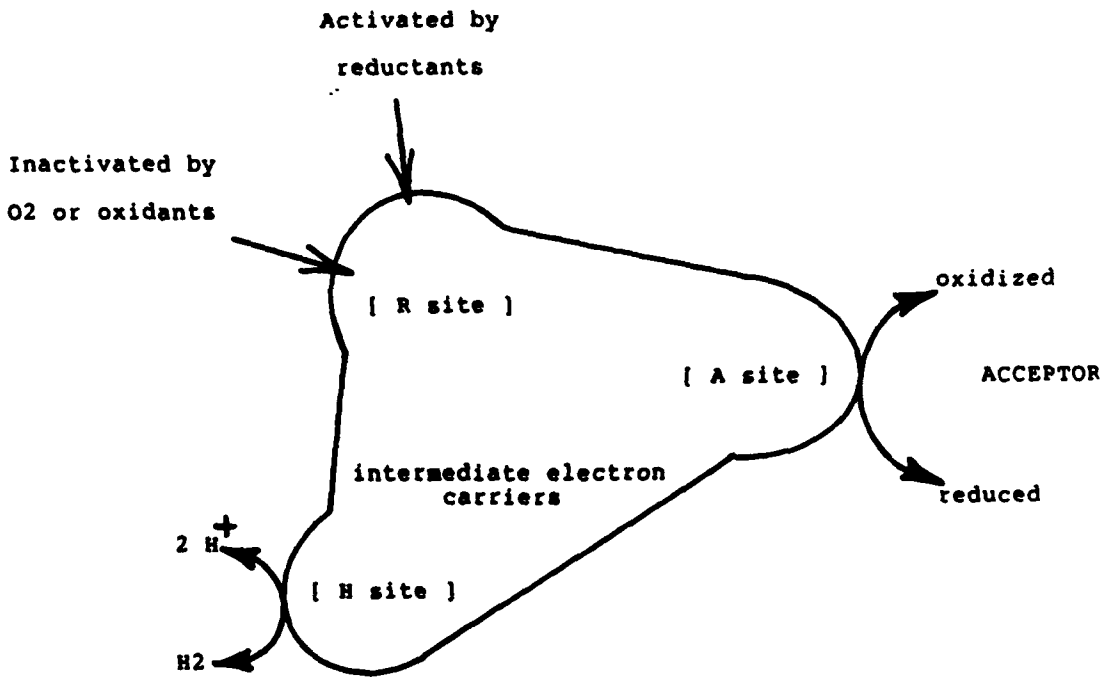

Figure 4. Cammack's (57) model for Hydrogenase molecule: The model depicts three functionally different domains to explain the diversity, mode of action and oxygen sensitivity of hydrogenases.

1. H-DOMAIN (Hydrogen activating site): Since all hydrogenases use the substrate hydrogen, they all must contain site for hydrogen binding. Components of this site may include the Fe-S clusters and other metal ligands, such as Nickel and Selenium.

2. A-DOMAIN (Acceptor site): As hydrogenases use a wide array of different electron acceptors (such as NAD^+, cytochromes, flavodoxin, ferredoxin etc) a somewhat complex acceptor domain is assigned to the molecule. This site naturally will have the greatest diversity among hydrogenases.

3. R-DOMAIN (Regulatory site): To account for the mechanism of reversible and irreversible inactivation of hydrogenases under reducing and oxidizing conditions, a regulatory site which is different from H-Domain is postulated. Although removal of oxygen is a pre-requisite for activation of the enzyme, it appears that oxygen does not bind to the H-site but rather interact with the R-domain and changes the conformation of the molecule and prevents H_2 binding at H-domain. Oxygen-free radicals-induced irreversible inactivation probably occurs at H-site.

substrate-derived electrons through a long respiratory chain to generate metabolic energy with great efficiency. In spite of its beneficial effects, oxygen also excerts detrimental effects on all organisms, especially anaerobic organisms, and even the strictly aerobic bacteria and higher organisms suffer from oxygen damage (58). For this reason, aerobic organisms have developed various defense mechanisms to cope with oxygen toxicity. The aerobic hydrogen-oxidizing and the nitrogen-fixing bacteria are particularly susceptible to oxygen partial pressure during growth. The two critical enzyme systems - hydrogenase and the ribulose biophosphate carboxylase - that are involved in the metabolism of hydrogen-oxidizing bacteria (so far studied) showed oxygen sensitivity to varying degrees. Adams et al. (17) have suggested that there is a correlation between the O_2-sensitivity of a particular enzyme and its physiological role, so that only those enzymes which normally catalyze H_2 production are very O_2-sensitive while others are generally less sensitive. Two types of oxygen-inactivation of hydrogenases have been proposed; oxygenation in which oxygen is bound to the enzyme and therefore reversible, and oxidation of a functional group and therefore irreversible. The chemical basis for these reactions are not well understood, though the formation of inactive oxygen products, destabilization of the iron-sulfur cluster and formation reactive oxygen species, such as superoxide anion (59,61) have been implicated in these processes. Electron paramagnetic resonance spectroscopy of various "oxygen-stable" hydrogenases (60) produced only inconclusive results as to the mechanism of O_2-inactivation. New evidence (57,60,61) indicates that the reversible inactivation of the enzyme is dependent on the environment, duration of O_2 exposure and reaction conditions, such as the type of catalytic mode the enzyme exits. Certain hydrogenases are sensitive to oxygen in both catalytic mode and non-catalytic mode while others are only in the catalytic mode. To explain some of these contradictions, Cammack et al. (56) introduced a general model for hydrogenase enzyme molecule (Figure 4) and postulated that oxygen binds to the regulatory site of the enzyme and gives a catalytically inactive conformation to the molecule. In this state oxygen acts as a stabilizer in the sense that the binding of oxygen prevents the enzyme from being converted into the catalytically active conformation, and that the hydrogen activation site (Fe-S cluster) of the molecule is left intact. In the presence of a reduced electron carrier or

reductant, the enzyme is converted into the catalytically active conformation (8,61,62) and is rapidly reduced by hydrogen. In this catalytic mode, many so called 'oxygen-stable' hydrogenases become very oxygen-sensitive. In contrast, the activity of the oxygen-stable soluble hydrogenase of *A. eutrophus* is not inhibited by oxygen (61) in the catalytic mode. It is of great interest to note at this time that this enzyme, unlike all other hydrogenases so far studied, is also insensitive to carbon monoxide (63). This enzyme can be purified and handled in air while they are inactive, and can be deoxygenated/reactivated without affecting activity.

2.4. Hydrogenase: Bio-technological applications.

The unique ability of the hydrogenases in reversibly activating molecular H_2 lends itself to a variety of practical uses. Since the discovery of photoproduction of H_2 from water by the coupling of hydrogenase with chloroplasts (26,27,55), researchers have been intensely exploring numerous biotechnological application of this enzyme. Solar energy conversion and storage, production of special chemicals using H_2 as the ultimate reductant, biological co-factor regeneration by immobilized hydrogenase, and many more such biotechnological applications are being attempted and pursued. It is obvious that for every process in which H_2 gas is consumed or evolved, the use of hydrogenases is worth considering, because it allows H_2 activation with minimum energy. Although there is much to be learned about the catalytic activities of hydrogenases at high pressure and about the immunological response that their "in vivo" administration may provoke in the mammalian system, it is worth taking the bold step of considering the use of injected hydrogenase to reduce the incidence of DCS after H_2 diving. A number of new bioengineering techniques, such as enzyme conjugation (49) or encapsulation may be employed to introduce the enzyme into the mammalian system without reducing its catalytic activity 'in vivo'. Genetic engineering of the catalytically active component of the enzyme might enhance its activity and reduce any undesirable side effects of in vivo administration.

2.5. Choice of *A. eutrophus* as the enzyme source.

Among the several aerobic hydrogen oxidizing bacteria that are potential source of the hydrogenase enzyme, we have chosen *Alcaligenes eutrophus* H16 over other organisms for many critical and practical considerations, foremost among them being the exceptional O_2-stability and carbon monoxide insensitivity of the soluble hydrogenase isolated from this bacteria. In addition:

1. This bacteria is a small (0.7 x 1 μ), Gram-negative coccobacillus of no known pathogenic property.

2. It can be grown in high rates and yields either autotrophically in the presence of H_2, or heterotrophically in the absence of H_2 in relatively simple media.

3. A wealth of information on its potential biotechnological application is available due to the interest NASA has shown in exploring the use of this microorganism as a bioregenerative system for distant space-flights.

4. Hydrogenases from *A. eutrophus* have been isolated and studied extensively and much information is available about their enzymatic and structural characteristics. Some of the recent advances in hydrogenase genetics (66,67) and the discovery of plasmids (28,50) in *A. eutrophus* that may code for enzymes of H_2 activation/CO_2 fixation make this organism ideally suited for bioengineering manipulation and practical application such as in the hydrogen diving.

3. EVALUATION OF TECHNIQUES FOR MASS-PRODUCTION OF *A. eutrophus*.

3.1. Statement of the problem.

A. eutrophus, as any other aerobic H_2-oxidizing bacteria, is a facultative autotroph and can grow under a wide variety of conditions, either chemolithotrophically or heterotrophically. It is, therefore, not surprising that numerous techniques for its culture under laboratory conditions have been reported in the literature. Therefore, the question was to select a method that offered the best chance for an easy scale-up, in order to harvest large amounts (several kilograms) of bacteria, which will yield enough purified enzyme for use in the proposed research project. Cost-effectiveness, safety concerns, and

availability of bacteria on demand were among some of the criteria in making the selection. For mass production, several researchers employed chemolithotrophic batch technique, by continuous bubbling of mineral medium with a H_2-O_2-CO_2 gas mixture to grow *A. eutrophus* autotrophically. Using this procedure on a rather large scale, Repaske and Meyer (7) reportedly obtained 25 g of dry cells per liter of culture medium. Scaling-up such a technique, however, would have required a special facility to meet the stringent safety requirements involved in the handling of large flows of H_2 gas mixtures. Anticipating possible delays and prohibitive costs in growing the bacteria autotrophically on a large scale, alternate methods were considered. Two techniques were evaluated and compared: the electrolytic chemolithotrophic method described by Schuster and Schlegel (5) and the fructose-glycerol heterotrophic method of Friedrich et al. (4).

3.2. Materials and methods.

Preparation of a sufficient volume of high density cell suspension grown under autotrophic condition is a prerequisite for electrolytic or heterotrophic growth of *A. eutrophus*. The methods described below for the preparation of the inoculum and for the assay of the activity of the enzyme in whole cells are common to both procedures.

a) Preparation of inoculum.

Two vials of lyophilized *A. eutrophus strain* H16 (ATCC 17699) were suspended in 2-3 ml of sterile mineral medium (1,5, and Appendix 2 for composition of the media). After allowing 3-4 h for hydration, the bacterial suspension was transferred to a 500-ml Erlenmeyer flask containing 100 ml of the same medium, which was previously saturated with a mixture of H_2(80%)-O_2(10%)-CO_2(10%). For preparation of the gas mixture, a 2-vessel gasometer system as depicted in Figure 5 was employed.

About 5 l of gas were prepared at a time and then bubbled through the 100 ml of medium, over a period of 15 min. This bubbling was generally repeated twice. A 0.2 μ filter was incorporated into the line to sterilize the gas mixture. After bubbling, the flask was tightly sealed and incubated in a shaking water-bath at 30 °C for 16-20 h or until the optical density at 436 mμ reached a minimum value of 1.0

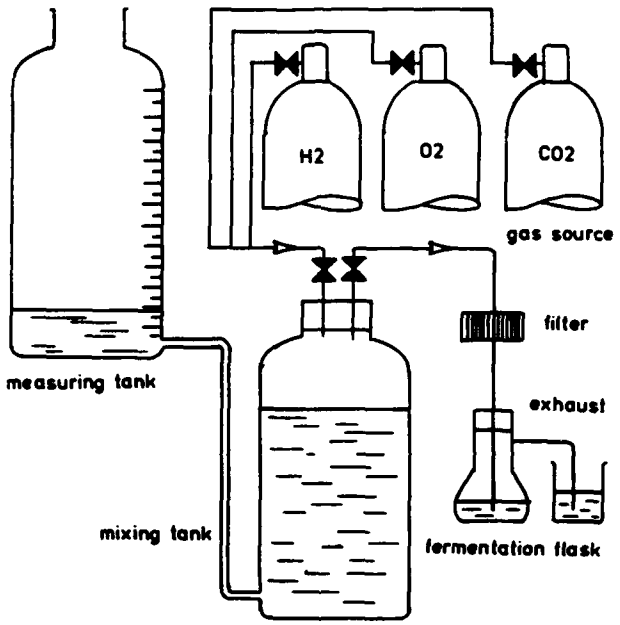

Figure 5. 2-Vessel gasometer system for preparing H_2-O_2-CO_2 mixtures: Measured volumes of H_2, O_2 and CO_2 are sequentially injected into the mixing vessel by water displacement. Once the desired composition of gas mix is obtained it was bubbled at a slow rate through the medium, using the hydrostatic pressure in the water column as the driving force.

and the bacteria passed the mid-log growth phase. The preparation was immediately used to inoculate the electrolytic or heterotrophic culture media (described below). The inoculum prepared by the above procedure remained viable up to 2 months when stored at 0-4 °C under sterile conditions. The purity and homogeneity of the bacteria was routinely checked by standard staining for gram-negative bacteria.

b) Assay of the soluble hydrogenase activity in whole cells.

This assay was performed by the NAD reduction method (8). After determining the protein content of washed cells, they were lysed by the addition of 0.005% hexadecyltrimethylammonium bromide and the enzyme activity was assayed spectrophotometrically by recording the reduction of NAD at 340 mμ. In a typical assay, a reaction mixture of 3 ml contained about 2.90 ml of 50 mM Tris-HCl, pH 8.0, previously saturated with pure H_2, 1 mM NAD^+ and about 100-200 μg of cell proteins. The reaction was carried out at 30 °C in a bubble-forming anaerobic Thumberg Cell [Precision Cells Inc. (12)] and the reaction was usually initiated by addition of 20-40 μl of enzyme from the side arm of the anaerobic cell. A unit of enzyme activity was defined as the reduction of 1 μm NAD^+ per minute per milligram of protein.

c) Chemolithotrophic electrolytic technique.

A. eutrophus can grow in a simple mineral medium containing a nitrogen source, potassium, magnesium, calcium, phosphate, sulfate, bicarbonate, traces of iron and nickel, and dissolved gaseous components such as H_2, O_2 and CO_2 (1,5). Numerous studies have been done to define the conditions for optimal growth for this bacteria by varying the composition of the mineral medium (2,3,6). In particular, an absolute requirement of nickel was found for obtaining active growth of A. eutrophus (4), but requirement for other trace metals such as cobalt etc. are not well ascertained. Another important consideration was the rate of flow and composition of the gas mixture as was recommended by previous investigators (7,8). Schuster and Schlegel (5) have conceived an elegant system to cultivate *A. eutrophus*, using gaseous components (H_2/O_2) produced by electrolysis of the mineral medium in a chemostat. The system was then bubbled with 10% CO_2 in N_2 to provide a carbon source and to maintain the pH. This chemostat was also employed to study the characteristics of steady state growth of the bacteria by continuous circulation of fresh media while harvesting the cells at the same rate.

Here at the Naval Medical Research Institute, Dr. L.A. Kiesow has built (with the superb technical help from William Mints) and tested a pilot unit (Figure 6) similar to the Chemostat of Schlegel (5). This unit was available for our initial investigation.

The 3-liter fermenter consisted of a microcarrier spinner flask (Belco Biotechnology, NJ) fitted with two large stainless steel electrodes through the side-arm screw caps. The center-neck screw cap was machined to accept a YSI O_2 electrode and two separate inlet and outlet ports for flushing the flask with a mixture of CO_2-N_2. The fermenter electrodes were attached to a power source that was able to deliver a maximum current of 1 ampere under fixed voltage. The O_2 electrode was attached to a YSI O_2-meter, the output signal of which was fed into a control unit. The electrolytic current was controlled by this unit to an intensity directly proportional to the difference between the O_2 pressure in the medium and a preset value of 0.25 atm. The O_2 pressure and the electrolytic events were continuously monitored on a 2-channel ink recorder.

Before initiating electrolysis, the spinner-flask containing 2.5-2.6 liters of the sterile mineral medium was incubated in a water bath at 30 °C and stirred at a rate of 500-600 rpm. The medium was then flushed with a sterile mixture of CO_2-N_2 (2%-98%) at a flow rate of 15-20 cc/mt. When the medium reached bath temperature, 100-200 ml of the autotrophically grown inoculum was injected asceptically through the gas inlet port (after temporarily disconnecting it) and the power source was switched on. Recurring electrolytical events were generated in the chemostat by keeping the O_2 pressure below the preset value of 0.25 atm by constant flushing of medium with N_2-CO_2 current. The growth of bacteria was periodically monitored by measuring the turbidity of the culture at 436 mμ.

d) Heterotrophic (fructose-glycerol) technique.

A. eutrophus, like other H_2-oxidizing bacteria could be grown in the absence of H_2 in a culture medium containing various organic compounds such as fructose, pyruvate, succinate etc. as energy and carbon sources (9,10,11) but the appearance of specific enzymes of autotrophic metabolism in heterotrophic growth depends on the nature of the organic substrate, and varies with organisms. The degree of synthesis of key enzymes (particularly hydrogenase) of autotrophic metabolism in hydrogen

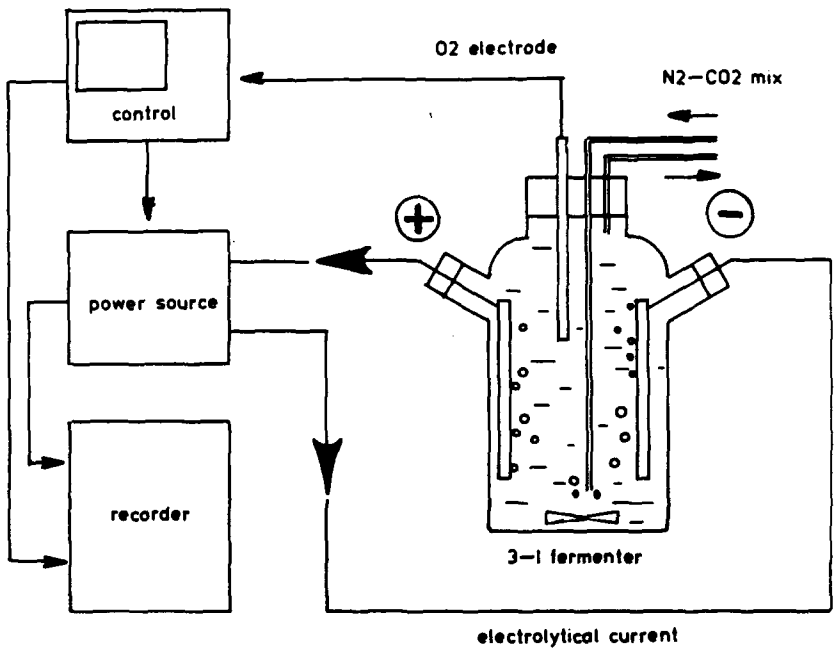

Figure 6. Electrolytic fermenter for growing *A. Eutrophus*: H_2 and O_2 are generated by electrolysis of the mineral medium. The bacteria recombine them into water and liberate energy for growth. Since the production of H_2 and O_2 are stoichiometrically related, monitoring and controlling the O_2 pressure is sufficient to operate the chemostat. The current is regulated proportionally to the difference between the observed O_2 pressure and a preset value (typically 0.25 bar). Additionally a N_2-CO_2 mixture was continuously flushed through the fermenter.

bacteria also vary with organism and organic substrate (9). For *A. eutrophus*, Friedrich et al. (4,10) designed a heterotrophic culture condition that allowed optimal expression of both soluble and membrane-bound hydrogenases using a fructose-glycerol medium (composition of this medium is given in Appendix 2). With the exception of the presence of 0.5% fructose, 0.2% glycerol, and 8 μm nickel, this medium displays only minor modifications in composition from the mineral medium used for chemolithotrophic growth.

For medium scale culture of the bacteria in the laboratory, 15 liters of this medium was prepared, sterilized through a millipore Millidisk filtering system fitted with a 0.22 um Durapore membrane cartridge and collected in a sterile incubation vessel (20 liter). The cap of the vessel (Figure 7) was fitted with inlet and outlet ports for flushing the medium with sterilized fresh air, and a central port for a stainless steel impeller that is connected to a rheostat-controlled motor. This assembly was placed in a water bath maintained at 30 ° C and flushed continuously with sterile air (150 ml/min) while stirring at 600 rpm.

About 100-200 ml of autotrophically grown bacteria was inoculated through the air inlet port and the aeration and stirring continued. Bacterial suspension was drawn periodically to ascertain turbidity due to growth and to assay the enzyme activity of the soluble hydrogenase. At the end of the run (generally after 50-55 h) the bacteria were harvested by centrifugation. The pooled bacterial cells were washed with cold potassium phosphate buffer (50 mM, pH 7.0) and kept frozen at -60 ° C for further processing.

3.3. Results.

a) Electrolytic method.

A typical recording of the functioning of the chemostat is presented in Figure 8. At about every 2 min an electrolytic event took place and consequently O_2 and H_2 were generated in the medium. When the O_2 pressure reached the maximum pre-set value at the control unit, the current input was automatically turned off but was turned on again with the subsequent decrease in O_2 pressure (due

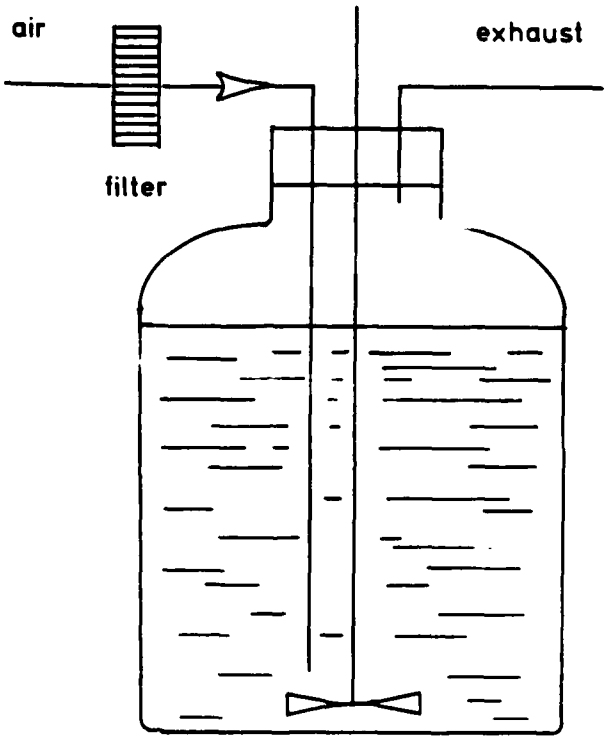

Figure 7. 20-l Fermenter for heterotrophic growth of *A. eutrophus*: 15 liters of mineral media containing 0.5% (w/v) fructose, 0.2% (w/v) glycerol and 200 ml of autotrophically grown *A. eutrophus* were incubated in a water bath at 30 °C. A constant stream of filter-sterilized air was bubbled through the medium while it was being stirred at 600 rpm.

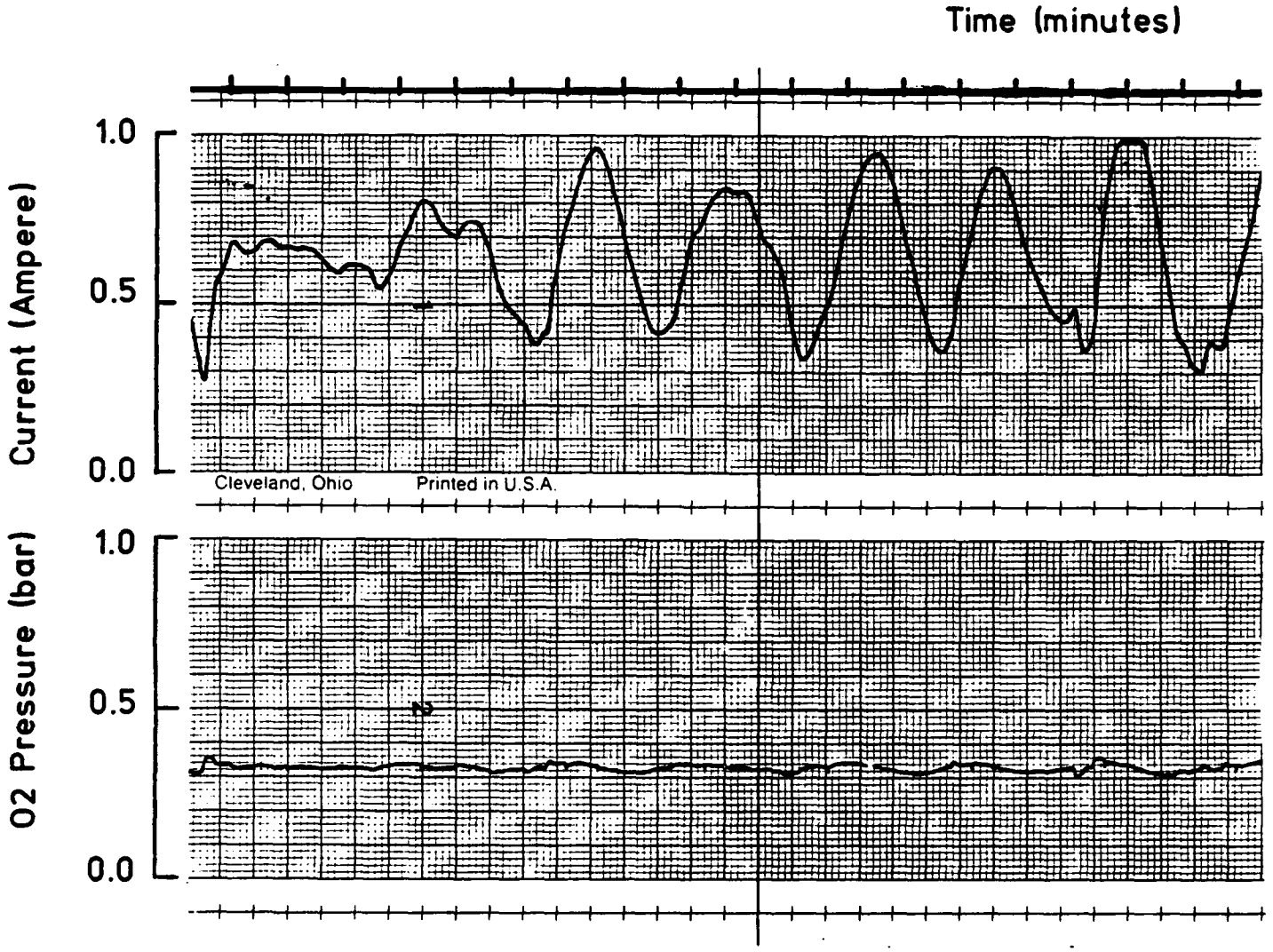

Figure 8. Typical electrolytic events as recorded on a two-channel recorder during electrolytic culture of *A. eutrophus*: Top portion is the profile of input current and the bottom is the O_2 pressure in the chemostat. Deviations from the preset O_2 value (0.25 bar) trigger electrolysis and generate H_2 and O_2 for bacterial growth.

partly to its consumption by bacteria and partly due to its elimination from the fermenter by N_2-CO_2 flushing).

During four independent runs by the above procedure, bacteria continued to grow without reaching a stationary phase over a 3-day period. However, it took about 15-20 h for the bacteria to reach an apparent mid log phase (Figure 9).

When two runs were allowed to continue beyond three days, the sensing ability of the O_2 electrode became severely impaired due to deposition of bacteria on electrode membrane. Thus unattended operation of the system became impossible. Attempts to run the system in the continuous mode were unsuccessful. Bacteria harvested after 80 hours of growth showed a cell concentration of 0.6 g/l and a soluble hydrogenase activity of 1-2 units per mg protein.

b) Fructose-glycerol method.

In the heterotrophic method, *A. eutrophus* grew logarithmically until about 10-12 h. Thereafter the growth rate was retarded, but the specific activity (activity per unit weight of protein) of soluble hydrogenase, which was absent or very low during the logarithmic growth phase, steadily increased in spite of the slow growth rate (Figure 10). The average yield of bacteria by this method was about 1.5 g/liter or about 20-25 gm per batch (wet weight). The average specific activity of the soluble hydrogenase from harvested cells was three units or more.

3.4. Discussion.

a) Electrolytic method.

Our attempts to grow *A. eutrophus* using the electrolytic method showed the importance of maintaining a high rate of flow of CO_2-N_2 gas through the culture medium during the entire operation. The CO_2 serves the dual purpose of furnishing the bacteria the carbon source and buffering the culture media through CO_2-bicarbonate system for effective pH control. Although there exists a stoichiometric relationship between the liberation of H_2 and O_2 during electrolysis of water and their consumption by the bacteria thereafter, a progressive enrichment of O_2 relative to H_2 occurred in the fermenter because

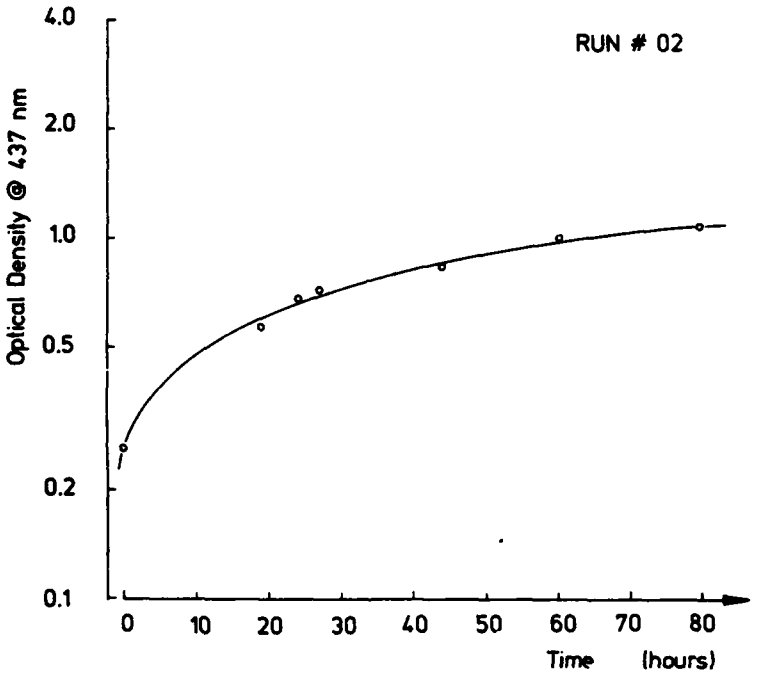

Figure 9. Growth curve for *A. eutrophus* in an electrolytic fermenter: The bacteria was grown, as described in the text, in a 3 liter chemostat which was continuously flushed with a mixture of CO_2 and N_2. Growth was followed by measuring the turbidity at 436 mμ at indicated time and the values were plotted semilogarithmically. An optical density of 1.0 roughly corresponds to 0.5 gm bacteria per liter.

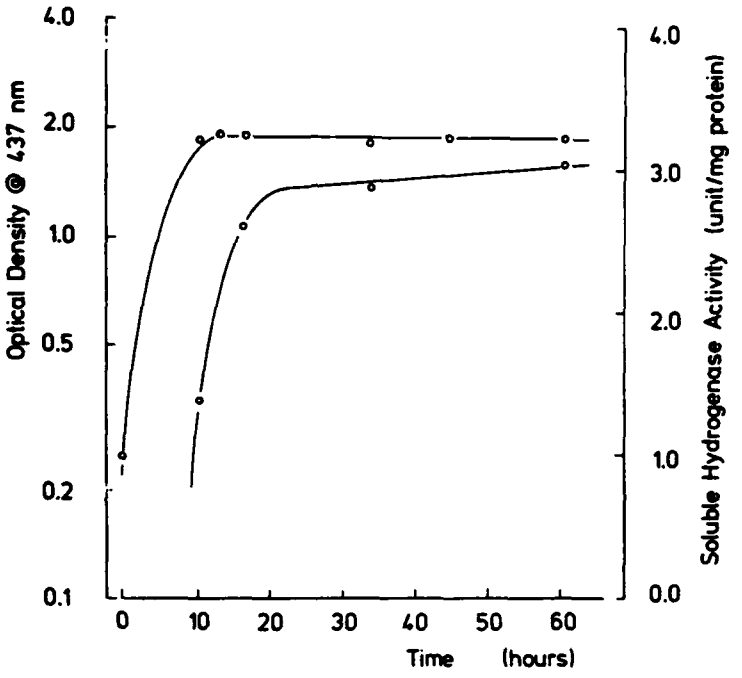

Figure 10. Hydrogenase activity in cells of *A. eutrophus* grown with fructose and glycerol as carbon sources: Cells were grown in minimal salt medium with 0.5%(w/v) fructose and 0.2% (w/v) glycerol. Growth curve is depicted as a semilogarithmic plot (o). Cells were drawn at indicated time intervals and the soluble hydrogenase activity (•) in whole cells were assayed by NAD-reduction method as described in the text.

of loss of H_2 from the vessel due to its negligible solubility in the medium, as well as its rapid diffusibility. Thus, increasing the CO_2-N_2 flow tends to eliminate the O_2 build-up and brings its concentration well below the preset value of 0.25 atm, allowing automatic but intermittent electrolysis and continuous bacterial growth. Our results also showed that the enzyme activity (1-2 units) of soluble hydrogenase after 80 h of growth was better than what was reported (4) for autotrophically grown A. eutrophus. In terms of yield of bacteria, however, the 0.6 g/liter was smaller than expected and was found unsatisfactory. Attempts to grow and harvest the bacteria by continuous mode was also proved unsuccessful. Though the electrolytic method (either batch or continuous mode) looked theoretically attractive and has minimal safety hazards, the observed low yield of bacteria alone justified to explore alternate techniques before attempting to scale-up the electrolytic method for mass production.

b) Heterotrophic method.

It was reported that maintenance of heterotrophic cultures of H_2-oxidizing bacteria occasionally resulted in the complete and irreversible loss of its ability to oxidize H_2, due to the inadvertent selection of mutants, the origin of which was found to be independent of the culture procedure (29). Since the frequency of such mutations was unknown, the inoculum in the present study was prepared in strict autotrophic conditions in the presence of H_2-O_2-CO_2 mixture, thus eliminating the likelihood of producing mutant organisms in the heterotrophic culture medium.

Our data on heterotrophic batch method were consistent with the original findings of Friedrich et al. (4,10). The cells grew rapidly (with a doubling time of about 145 mts) until fructose was depleted from the medium. Thereafter, the growth significantly slowed down due to a possible substrate downshift to glycerol (glycerol is a poor carbon source for *A. eutrophus*), but the activities of the soluble hydrogenase (and of the membrane-bound enzyme) increased dramatically during the transition from fructose to glycerol. Substrates such as fructose, pyruvate, succinate etc., which support fast growth of hydrogen bacteria, exerted a severe repression or inhibition of synthesis of certain enzymes of autotrophic metabolism but they were derepressed during substrate shift to such poor carbon sources as fumarate, glycerol, etc. (10). The regulatory mechanisms involved in this derepression phenomenon,

including that of hydrogenase enzymes, were not well understood. The relatively high concentration of nickel (about 8 μm) added to the medium in the current procedure possibly contributed to an enhanced synthesis of the enzyme, as was noted by others (65). Nevertheless, the hydrogenase activity obtained from the current cell preparations was less than what was reported by Friedrich et al. (4). Further refinement of the culture technique was undertaken to enhance the activity of the enzyme.

c) Comparison of the two methods.

The electrolytic method is very elegant since it replaces the need for bubbling the mineral medium with the explosive H_2/O_2 mixture. Instead, this gas mixture was electrolytically generated in the medium itself, at a concentration sufficient enough for the optimal growth of the organism. The method involved essentially the autotrophic growth conditions, but the instrumentation and technique were rather complex and time consuming, and the yield was very low. The method had several drawbacks that seemed to prohibit its adoption for mass-scale production of the bacteria. The heterotrophic technique, on the other hand, was less complex, and avoided the use of the inflammable H_2/O_2 mixture entirely; it was therefore the least hazardous method. Only simple equipments and minimal technical skills were required to operate the heterotrophic culture system. Besides, both the quantity of bacteria harvested per unit volume of medium and the hydrogenase activity found per unit mass of protein were significantly higher in the heterotrophic method than in the electrolytic method.

d) Scale-up and mass production.

A significant advantage of the heterotrophic method was that direct scale-up of the method was feasible using a standard industrial fermenter (40-300 liter capacity), while the electrolytic method would require the construction of an altogether new and complex fermenter, with sizeable financial investment. In the former method, bacteria can be grown to predictable quantities by controlling the fructose concentration and the size of the fermentation vessel.

Following our pilot studies, the heterotrophic method was successfully adapted for large scale cultivation of the bacteria at the Bio-Scale-Up Facility of the University of Maryland. In accordance with our specifications, under a U.S. Navy contract, this facility grew *A. eutrophus* in fructose-glycerol

medium in a 35-liter standard fermenter and obtained about 10 g/liter of bacteria. Production of bacteria, using an industrial fermenter (300-liter capacity) at the same facility was envisaged, and should produce large amounts of cells that would be required to purify the soluble hydrogenase enzyme needed in the H_2 diving experiments at Naval Medical Research Institute.

4. CONCLUSION.

After examination of the characteristics of various species of H_2-oxidizing bacteria, it was established that *Alcaligenes eutrophus* H 16 (ATCC 17699) was the most suitable hydrogenase enzyme source for the feasibility study of biochemical decompression in animal, using H_2 gas mixtures. This choice was primarily based on the exceptional O_2-resistance of the soluble hydrogenase isolated from this organism and the ability of this enzyme to function under normal physiological conditions. In addition, the biochemical and immunological properties of this enzyme have been extensively studied and there is an ongoing interest of its use as a versatile biotechnological tool for several industrial applications. For these reasons, methods for growing *A. eutrophus* were reviewed with special consideration for its safe and economical large scale production. Two methods that did not involve handling of large quantities of the explosive H_2-O_2 mixtures were studied under laboratory conditions and the results were compared. After careful evaluation of the results of these pilot studies, a heterotrophic culture procedure (using fructose and glycerol) was selected and successfully tested for large-scale production at an external facility through a private contractor.

The fulfillment of these two milestones of the research project allows NMRI's investigators to proceed to the next phase of the research: the isolation of pure hydrogenase enzyme in sufficient amounts, tolerance studies of the enzyme in mammalian system, and development of techniques to evaluate and quantitate the enzyme activity in vivo.

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APPENDIX 1

A comparison of some properties of hydrogenases from representative bacteria.

		Molecular mass (kD)	Subunit(s) mass (kD)	Fe atoms/ molecule	S atoms/ molecule	Ni atoms/ molecule	Se atoms/ molecule	Type of (Fe-S)	Physiological e-carrier	Hup or Hpr	O2 lability	N2-fixing	Specific activity (U/mg)	References
Alcaligenes eutrophus H16	MB	98	67,31	6	6	0.7	-	(3Fe-xS) (4Fe-xS)	Quinon	Hup	(-,+)	-	-	12,22
" "	SOL	205	68,60,29 29	12	12	2.0	-	2(4Fe-4S)FMN 2(2Fe-2S)	NAD+	Hup	Stable	-	48	13,23
Nocardia opaca 1b	SOL	200	64,56,31 27	14	14	4.0	-	4(4Fe-4S)FMN 2(Fe-2S)	NAD+	Hup	(-)	-		52,53
Pseudomonas pseudoflava	MB	98	65,30	6	6	-	-							14,33,35
Methanobacterium thermoautotrophicum		60	60	38	27	3	2							55
Methanococcus vannielii	MB	131	42,35,27 27				4							61,62
Rhodopseudomonas capsulata	MB	65	65	3.6	3.6	1	-							64
Clostridium pasteurianum	SOL	60	60	12	12	-	-	3(4Fe-4S)	Ferredoxin	Hpr	(++)		4000	1,41,56,67
Megasphaera elsdenii	SOL	50	50	12	12	0.4	-	3(4Fe-4S)	Ferredoxin	Hpr	(+)		8400	2,3,42
Desulfovibrio gigas	PERI	89	62,26	12	12	1.2	-	3(4Fe-4S)	Cyt.c	Hup	(+)		91	4,8,9,43-45
Desulfovibrio vulgaris Str. Hildenborough	PERI	50	50	12	12	0.7	-	3(4Fe-4S)	Cyt.c	Hup	(+)		10400	5,9,57
Str. Miyazaki	MB	89	59,28	8	8	-	-	2(4Fe-4S)	Cyt.c	Hup	(-)		90	6
Desulfovibrio desulfuricans	MB/SOL	52	52	12	12	1.0	0.5				(-)		9000	7,58,59,68
" " -27774	SOL	78	39,39	11	11	1.0	-				(+)			60
Escherichia coli	MB	113	56,56	12	12	-	-			Hpr	(+)			10
Proteus mirabilis	MB	205	63,63,33 33							Hpr	(-)		low	11

A comparison of some properties of hydrogenases from representative bacteria. (Con't)

		Molecular mass (kD)	Subunit(s) mass (kD)	Fe atoms/ molecule	S atoms/ molecule	Ni atoms/ molecule	Se atoms/ molecule	Type of (Fe-S)	Physiological e-carrier	Hup or Hpr	O2 lability	N2-fixing	Specific activity (U/mg)	References
Chromatium vinosum	MB	62	62	4	4	1.8	-	1(4Fe-4S)			(-)		42.5	15-17,46
Rhodospirillum rubrum	MB	66	66	4	4	-	-			Hup	(-)		33	18,47
Thiocapsa roseopersicina	SOL	68	47,25	4	4					Hup	(-)		5	19
Alcaligenes paradoxus	MB													24
Alcaligenes ruhlandii	SOL/MB								NAD					21
Alcaligenes latus	MB										(+)	+		48
Pseudomonas facilis	MB													32
Pseudomonas saccharophila	SOL/MB													20
Pseudomonas carboxydovorans	MB													69
Pseudomonas flava	MB													33,35
Pseudomonas palleronnii	MB													35
Pseudomonas hydrogenovora	MB													34
Pseudomonas hydrogenothermophila	MB													28
Pseudomonas thermophila	MB													30
Flavobacterium autothermophilum	MB													28
Azospirillum	MB					?								66
Hydrogenomonas thermophilus	MB													30
Aquaspirillum autotrophicum	MB													30
Paracoccus denitrificans	MB										(+)			31,49
Corynebacterium autotrophicum	MB													25
Nocardia autotrophica	SOL								NAD					30
Mycobacterium gordonae	MB													30
Arthrobact er sp. (11x,RH12)	MB													30
Seleberia carboxydohydrogena														30
Azospirillum lipoferum	MB					?			Ferredoxin			+		26,66
Derxia gummosa	MB					?						+		27,66
Microcyclus aquaticus	MB											+		29

A comparison of some properties of hydrogenases from representative bacteria. (Con't)

		Molecular mass (kD)	Subunit(s) mass (kD)	Fe atoms/ molecular	S atoms/ molecular	Ni atoms/ molecular	Se atoms/ molecular	Type of (Fe-S)	Physiological e-carrier	Hup or Hpr	O2 lability	N2-fixing	Specific activity (U/mg)	References
Mycrocyclus ebruneeus	MB											+		30
Renobacter vacuolatum	MB										(+)	+		30
Rhizobium japonicum	MB										(+)	+ +		27,51
Xanthobacter autotrophicus	MB											+ + +		36
Xanthobacter flavus	MB													38
Arthrobacter Str. 11/X	MB													37
Bacillus Schlegelli	MB										(+)			39
Acetobacter virelandii	MB										(+)			50
Azobacter chroococcum	MB					?								63
Vibrio succinogenes	MB													65
Azospirillum brasilense	MB					?								66

MB = Membrane-bound; SOL = Soluble and cytoplasmic; PERI = Periplasmic; KD = Kilodaltons; Hup = Hydrogen consuming; Hpr = Hydrogen producing; Cyto.c = Cytochrome C.

References for Appendix 1

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APPENDIX 2

COMPOSITION OF CULTURE MEDIA

A. Electrolytic Culture	B. Heterotrophic Culture
25 mM Na_2HPO_4	25 mM Na_2HPO_4
11 mM KH_2PO_4	11 mM KH_2PO_4
7.6 mM Ammonium Sulfate	37.4 mM NH_4Cl
0.8 mM $MgSo_4$	0.8 mM $MgSO_4$
14.5 uM $CaSO_4$	180 uM $CaCl_2$
5.3 uM $NiSO_4$	8 uM $NiSO_4$
0.1 mg% Ferric Ammonium Citrate	0.5 mg% Ferric Ammonium Citrate
6 mM Sodium bicarbonate	6 mM Sodium Bicarbonate
pH 7.0	0.5 % (w/v) Fructose
	0.2 % (w/v) Glycerol
	pH 6.8-7.0

Note: Inoculum for both culture procedures was grown in the electrolytic culture media.